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In vitro studies on tumor-specific immunity by using C3H mammary cancer-A cells. 3. Specificity of aggregation of sensitized lymph-node cells on target cells

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In vitro studies on tumor-specific immunity by using C3H mammary cancer-A cells. 3. Specificity of aggregation of sensitized lymph-node cells on target cells*

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Abstract

As a link in the series of studies on tumor specific immunity an attempt was made to clarify specificity if any, in aggregation of sensitized lymph-node cells on target cell in vitro. For this purpose sensitized regional lymph-node cells from isologous CsH mouse transplanted with A cells derived from CaH mouse mammary cancer were incubated with M cells derived from mammary cancer of homologous Cb mouse and HeLa-Ss cells as with A cells. The results are briefly summarized in the following. These sensitized regional lymph-node cells (A-L) inhibited the proliferation of A cells and M cells in tissue culture. When the interaction between the sensitized lymph-node cells and the terget cells was pursued over a long period by cinematography, these lymph-node cells became attached to the target cell by 6-to 12-hour culture in aggregation of rosette form, and by 30 hours some of the target cells were seen to undergo lysis. However, when these sensitized lymph-node cells were cultured with heterologous HeLa-S3 cells (derived from human uterine cancer), no such phenomena were observed. In the case with untreated normal lymph-node cells (control) there could be hardly observed any inhibitory effect on target cells. When the number of the target cells on which the lymph-node cells became attached was counted along with lapse of time, it was more numerous in the case of A and M cells but only a few in the case of HeLa-S3 cells. It seems that most of the sensitized lymph-node cells that inhibit the growth of the target cells become attached and aggregated fairly specifically onto the target cells.

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IN VITRO STUDIES ON TUMOR-SPECIFIC IMMUNITY BY USING C_3H MAMMARY CANCER-A CELLS

III. SPECIFICITY OF AGGREGATION OF SENSITIZED LYMPH-NODE CELLS ON TARGET CELLS

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After the transplantation of A strain cells (derived from C_3H mouse mammary cancer) to isologous C_3H mouse regional lymph-node cells obtained from the host at an early stage of inoculation show inhibitory effect not only on the proliferation of A cells *in vitro*[1] but also on that of M strain cells derived from homologous Cb mouse mammary cancer and JTC-11 strain cells derived from hybrid mouse mammary cancer. Further it has been demonstrated that the effect of the sensitised lymph-node cells in tissue culture reveals a considerable specificity as verified numerically by the rate of proliferation of tissue culture cells[2]. In addition, it is already known that such sensitized lymph-node cells aggregate around the target cells when they inhibit the proliferation of the latter[3,4].

The present report was conducted to see the effect of sensitized lymph-node cells on isologous, homologous and heterologous target culture cells *in vitro* from morphological view including the manner how these lymph-node cells become aggregated to the target cells and it was found that the contactual aggregation indicates species specificity at least in the contactual aggregation of these lymph-node cells on a single culture cell.

MATERIALS AND METHODS

Animals: Female inbred mice of C_3H ($H\text{-}2^K$) strain, age 6—8 weeks old, were used for the experiment.

Tissue culture cells: Three strains of culture cells, namely, A cells derived from C_3H mouse mammary cancer, M cells derived from Cb mouse mammary cancer, and HeLa-S_3 cells derived from human uterine cancer, served as the materials. All these culture cells were first treated with 0.25% trypsin GKN solution, and both A- and M strain cells were cultured in the medium containing 50% bovine serum plus YLE solution while Hela-S_3 cells in the medium containing 20% bovine serum plus YLE solution.

Sensitization of mice and the preparation of lymph-node cell suspensions: To C_3H mice 5×10^6 A cells are transplanted subcutaneously on the back between the scapulas and 10 days later lymph nodes of the axilla are taken out, and for the control the axillary lymph nodes of untreated normal C_3H mice are used. Lymph-node cells suspensions prepared with the respective group are designated as A-L and n-L. The lymph nodes taken out are cut into small pieces with ophthalmic scissors, small pieces are passed through the 80-mesh filter, the filtrates are washed three times with a cold Hank's solution by centrifugation at 2, 000 rpm for 5 min each, and after removing serum, each group of the cells is suspended in the medium optimal to each of the three tissue culture cells.

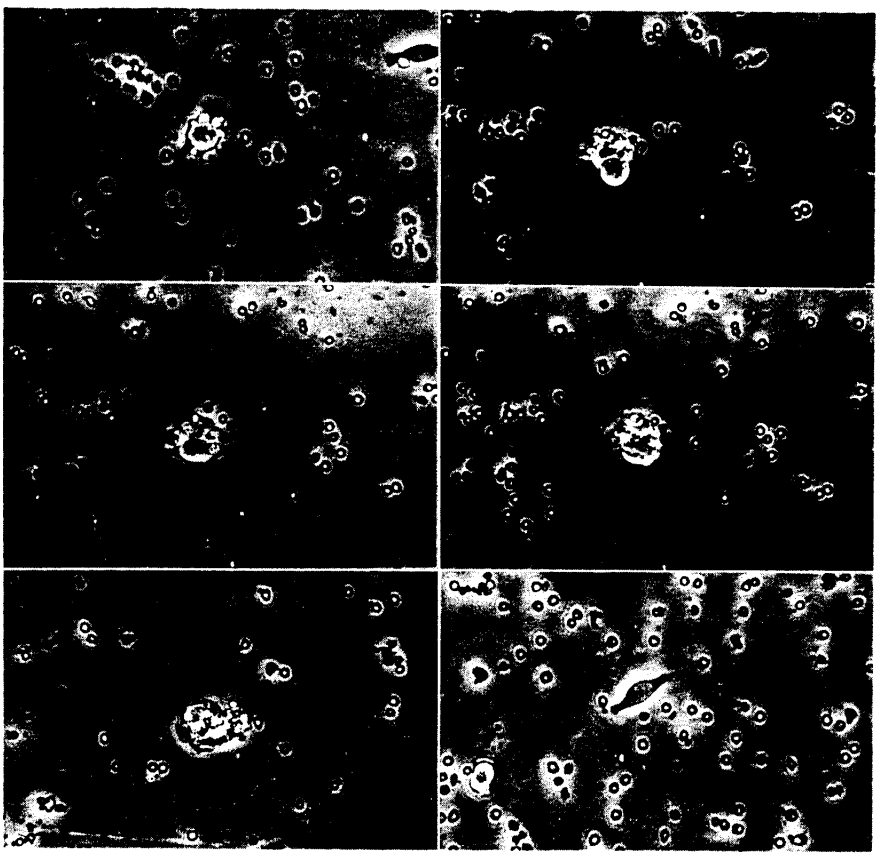
Culture of lymph-node cells and target cells: To a culture bottle, TD-15 with the wall of less than 0.6 mm in thickness, 4×10^4/ml of respective tissue culture cells and 16×10^5/ml of lymph-node cell suspension are placed and the mixed culture is carried out at 37°C.

Method of observation: TD-15 culture bottle is set in the phase-contrast cinematography apparatus ACM-2 model attached with Bolex H16 Reflex, and cinematographic pictures of 1 frame/20 sec are taken continuously for 60 hours. In this way changes in behaviors are recorded on the 16 mm RP negative films. Observations are also conducted at random of the entire view under low magnification and pictures are taken with 35 mm films and counts are taken of the number of aggregated lymph-node cells.

Those lymph-node cells that appear simply attached to the target cells are the ones shown in Photo 11 and they are distinguished from those cells in contactual aggregation as observed in Photo 12.

RESULTS

Observations of the cell behaviors by cinematography: The regional lymph-node cells obtained 10 days after the transplantation of A cells to isologous mice inhibited 40～50 per cent of the proliferation of A cells[1,2]. When the reaction of the lymph-node cells to A cells are observed by the phase-contrast time lapse cinematography, A-L lymph-node cells become attached around A cells by 6 to 12-hour tissue culture. Although some of them again leave the target cells, the number of such attached cells increases gradually. Some of the lymph-node cells are attached to the target cell with protrusions while others present a rossette formation. About this time granules in the cytoplasm of the target cell are still moving around. However, by about 30 hours the movement of both granules as well as of the wavy cell membrane of the target cell becomes sluggish and there occurs a sudden rupture of cytoplasmic membrane and A cells and some of lymph-node cells lose their mobility, and there can be observed no cell division even at 60-hour culture (Photos 1～5). Some of A cells aggregated



Photoplates show A strain cells in the mixed cell culture with regional lymph-node cells obtained from the C_3H mice 10 days after transplantation of 5×10^6 A strain cells. ×240 (1—5)

1: At culture hour 7, 2: At culture hour 16, 3: At culture hour 24, 4: At culture hour 27, 5: At culture hour 33, 6: At culture hour 6 (showing the mixed culture of A strain cells with untreated normal lymph-node cells)

with lymph-node cells do, however, divide into two cells by about 20-hour culture while the other without any lymph-node cells goes on dividing and multiplies by cell division (Photos 1, 2).

Such changes can also be observed in the case where M cells are cultured with A-L cells. However, in the case of the mixed culture of heterologous HeLa-S_3 and A-L cells there is seen no marked interaction. In the case of the control group where normal lymph-node cells (n-L) are cultured there can be observed none that become attached to or aggregated onto A cell or M cell, and the culture cells proliferate by cell division (Photos 6～10).

The number of tissue culture cells in contact with lymph-node cells aggregated on them as observed by 35 mm film at a low magnifications: When the number of those lymph-node cells simply attached to the target cell (Photo. 11) and those that are in contactual aggregation (Photo. 12) are plotted on the abscissa and the corresponding number of the target cells is on the ordinate, the columns as shown in Figs. 1～6 are obtained.

Looking carefully at the number of A-L and n-L (control) cells simply attached or attached in contactual aggregation to each target cell, the following results are obtained.

a) As illustrated in Figs. 1, 2, the number of lymph-node cells attached

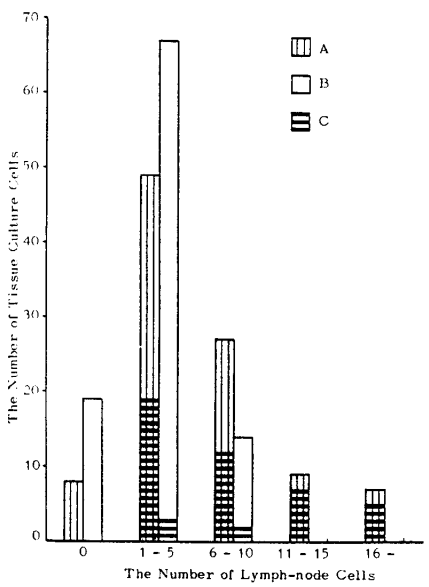
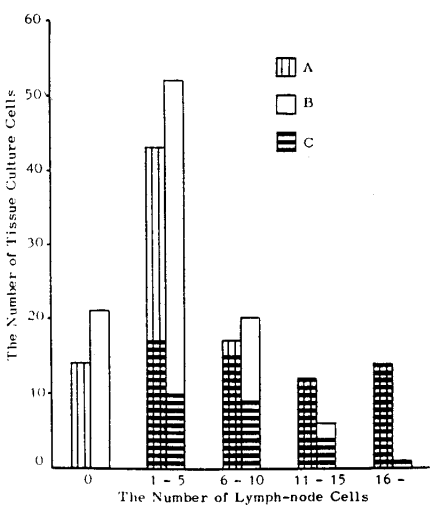
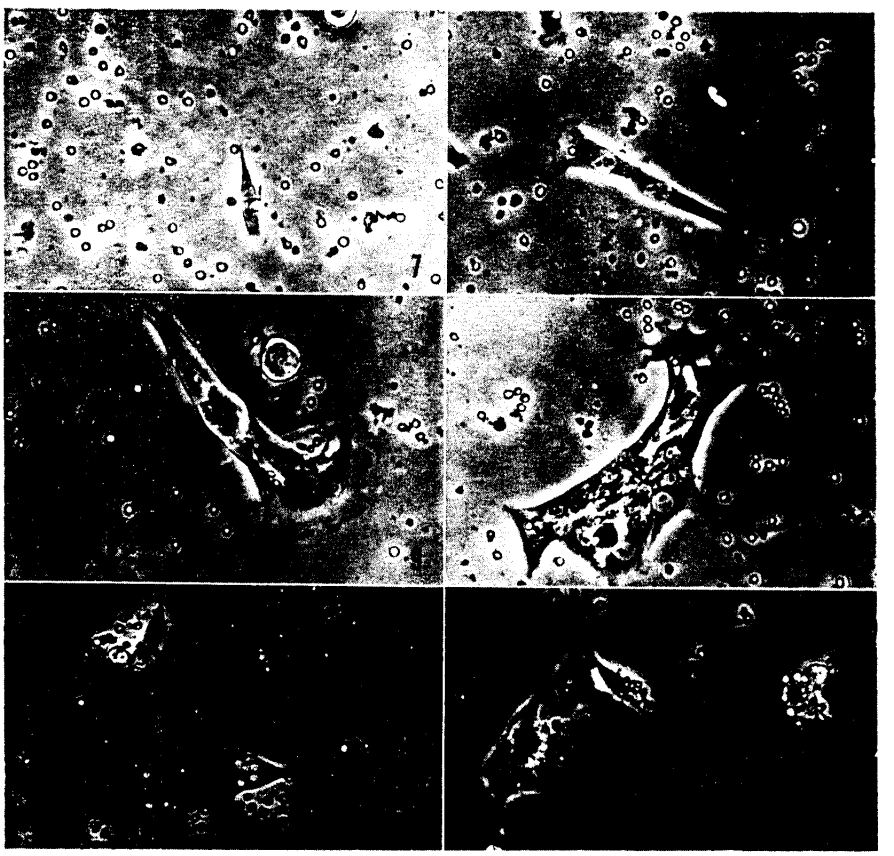


Fig. 1 Showing the number of the regional sensitized lymph-node cells attached to A cell and that in aggregate at 18-hour tissue culture
A: sensitized group, B: control group, C: group in aggregation



Fjg. 2 The number of the regional lymph-node cells attached to A cell and that in aggregation at 48-hour culture
A: sensitized group, B: control group, C: group in aggregation



Photoplates showing the mixed culture of A strain cells with untreated normal lymph-node cells. ×240 (7–11)
7: At culture hour 15, 8: At culture hour 24, 9: At culture hour 48, 10: At culture hour 60, 11: At culture hour 30, 12: At culture 48, (showing the mixed culture of A strain cells with regional lymph-node cells)

simply to the target cell does not differ materially between A-L and n-L as a whole, but in the case where more than six lymph-node cells are attached to one A cell the number is distinctly larger in those attached to the target cell in aggregated form.

b) In the case where the target cells are homologous M strain, the manner of attachment and aggregation of A-L cells to the target cell is similar as in the case of isologous A cells. In this instance, quite many cells of n-L are seen simply attached to the target cell but hardly any aggregation of these cells can be observed (Figs. 3, 4).

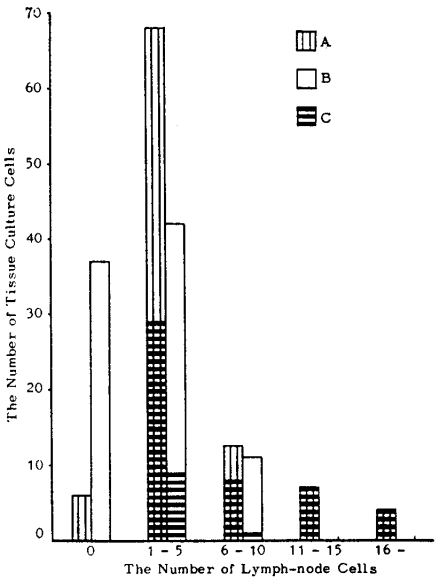


Fig. 3 The number of regional lymph-node cells attached to M cell and that in aggregation at 18-hour tissue culture
A: sensitized group, B: control group, C: group in aggregation

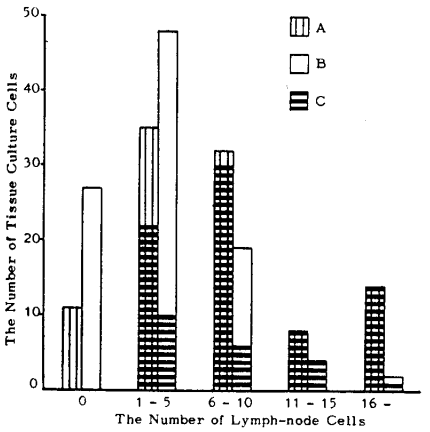


Fig. 4 The number of regional lymph-node cells attached to A cell and that of the cells in aggregation at 48-hour tissue culture
A: sensitized group, B: control group, C: group in aggregation

c) In the case of heterologous HeLa-S_3 cells as target cells, differing from the case where isologous or homologous cells are used, there is observed no difference at all between A-L and n-L even at 19 hours or at 48 hours of the mixed culture. Although there can be seen some cells attaching to the target cell, contactual aggregation of these cells is rarely observed (Figs. 5, 6).

It seems that lysis of 10 per cent or less has been elicited in the target cells (A strain or M strain) by the contactual aggrekation of A-L (Photo 5).

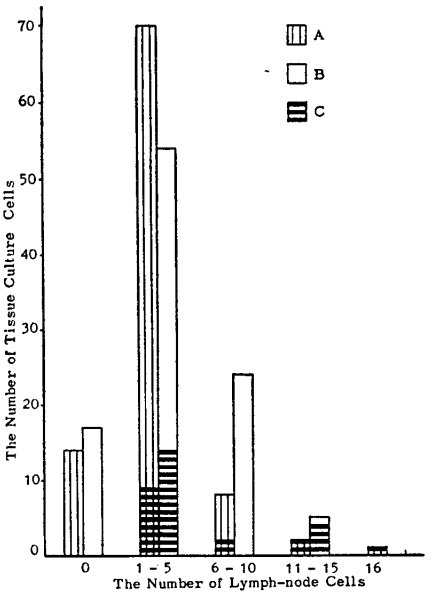


Fig. 5 The number of regional lymph-node cells attached to HeLa-S$_3$ cell and that in aggregation at 18-hour tissue culture
A: sensitized group, B: control group, C: group in aggregation

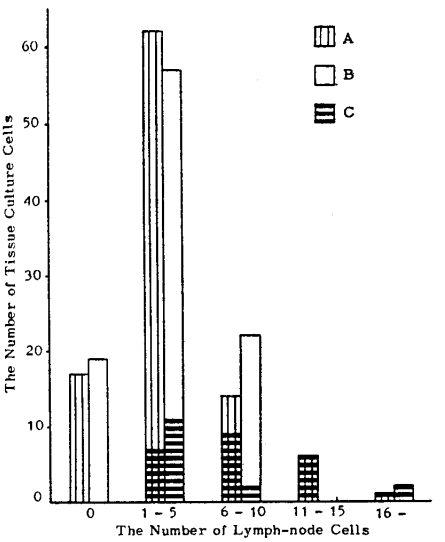


Fig. 6 The number of regional lymph-node cells attached to HeLa-S$_3$ cell and that in aggregation at 48-hour tissue culture
A: sensitized group, B: control group, C: group in aggregation

DISCUSSION

It was KOPROWSKI and FERNANDES[3] who first reported that sensitized lymphoid cells aggregate on their target cells in tissue culture, and in Japan HANAOKA[5] was the first to make such a report. Recently, HARA[6] found that the regional lymph-node cells from mouse transplanted with Ehrlich tumor aggregated on JTC-11 strain cells (derived from Ehrlich tumor) *in vitro* and these cells inhibited the division and proliferation of the target cells.

KOPROWSKI and FERNANDES[3] demonstrated that, when the lymph-node cells of the Lewis rat sensitized with guinea-pig spinal cord tissue in Freund's adjuvant were added to the tissue culture monolayer of the tissue of puppy brain, the lymph-node cells aggregated around glial cells bringing about the destruction of the glial cells, but fibroblasts survived. They gave the term, contactual agglutination to this phenomenon of lymph-node cells aggregating on glial cells. However, there is no precise explanation nor any fixed nomenclature for this phenomenon of sensitized lymph-node cells aggregating onto the target cell and bringing about the lysis of the latter. There is only a proposition of allergic death *in vivo* by BOYSE[7], and GORER and BOYSE[8]. Therefore, SATOH *et al*[9].

gave a tentative term of "immune adherent cell lysis" phenomenon (I. A. C. L. phenomenon) to this cell lysis *in vitro*.

The time when sensitized lymph-node cells aggregate on target cells varies greatly by the method of sensitization and conditions of tissue culture. TAYLOR[10] considers that these cell begin to aggregate onto target cells within six hours, and ROSE[11] around 18-hour culture. KOPROWSKI[3] stated that such an aggregation begins within one to three hours and the greatest number of these cells aggregated to one target cell at three-hour culture, showing 10～15 cells gathered on one target cell. Changes occurring in the target cell after the aggregation of sensitized lymph-node cells are also variously reported.

ROSENAU[12] reported that in his observations by the time lapse cinematography of the mixed tissue culture of the spleen cells from BALB/c mouse sensitized with L cells and L cells in proportion of 20: 1, one to three lymphocytes are found attached on the surface of the L cells, and these L cells withdraw their cellular projections and become rounded and vigorous movement of cytoplasm ceases, and the L cell, swelling up rapidly, shows the rupture of its cytoplasmic membrane. Simultaneously the majority of those lymphocytes attached to L cell loses cytoplasmic mobility, but such a phenomenon is not necessarily observed in all the L cells. VAINIO[13] found in his experiment with lymphoid cells from the mouse sensitized with allogenic tissue of different H-2 locus, embryonic fibroblasts as its antigen, that the lymphoid cells became aggregated around the target cell but even at 72-hour tissue culture lysis hardly occurs. However, in the case with fibroblasts of kidney or lung from adult mouse, being highly susceptible, the majority of the target cell is lysed within 24 hours, and the remnant antigenic cells become covered by aggregated lymphocytes, resulting in vacuolated cytoplasm.

TAYLOR[10] observed the interaction of L cells and sensitized spleen cells from homologus BALB/c mouse and heterologous guinea pig potently sensitized with L cells *in vitro*, and found that six hours after the addition of sensitized spleen cells the lysis of L cells began and it became more marked up to 72-hour culture, and those broken cells showed the swelling of mitochondria and the retraction of cytoplasmic processes. However, he did not mention about the swelling of the L cell itself. He further states that there occurs a subsequent disruption of the cell membrane and cytoplasmic granular material is released, and the nucleus becomes necrotic. These sensitized cells tend to aggregate around the damaged cells in clumps, and this aggregation is most marked in the period between 24 and 70 hours.

In contrast, in the present observations sensitized lymph-node cells aggregated around the target cells but about the time when lysis did occur, swelling of the cells could hardly be observed but rather atrophic picture was observed.

However, some of the cells that appeared to be dead become swollen and within a few minutes these cells were disintegrated as if by sudden explosion. What percentage of the inoculum size of tissue culture cells would be destroyed can be defined only by pursuing individual cells by time lapse cinmatography, but in the case of the counting by the phase-contrast microscopy, assuming the cell like the one shown in Photo 5 to be undergoing lysis, less than 10 per cent of the cells did undergo lysis.

Even in the case with untreated normal lymph-node cells some tissue culture cells were fonnd to have the lymph-node cells affached on them, but this seems to be related in part to the phenomenon of "peripolesis" as mentioned by SHARP and BURWELL[14]. HANAOKA[5,15] likewise observed that untreated lymdh-node cells only move around tissue culture cell in a close contact but they do not become attached to the target cell with their cytoplasmic processes. In the present experiment no phenomenon like "emperipolesis" as described by PULVERTAFT[16] could be seen.

Species specificity at least numerically could be discerned[2] in the growth-inhibitory effect of sensitized lymph-node cells, and morphologically many A-L cells aggregated around homologous M cells and isologous A cells, leading them to their lysis, but with heterologous HeLa-S_3 cells the aggregation of A-L was less and no lysis could be seen. In other words, species specificity seems to lie in this aggregation phenomenon. ROSE[11] likewise observed that when 2.7 million lymph-node cells of a rabbit sensitized with rabbit thyroglobulin were added to incomplete monolayer cells of rabbit thyroid gland, lymph-node cells were found attached to the target cells by 18-hour culture, and by 24 hours there were observed not only such adherence but also growth-inhibitory effect. However, when 2 million such lymph-node cells were made to challenge HeLa cells, no attachment could be seen, and with 20 million cells, there occurred adherence of Lymph-node cells but no growth-inhibitory effect.

While it seems that for the growth-inhibitory effect of sensitized lymph-node cells on the target cell *in vitro* this aggregation of lymph-node cells is essential, but even when sensitized lymph-node cells are placed in indirect contact with antigenic cells by enclosing the former cells in the diffusion chamber as done by HARA[17], since inhibitory effect on the proliferation of antigenic cells is observed, the aggregation phenomenon seems not to be indispensable. Consequently, it is natural that there is observed only a relative relationship between the number of the target cells inhibited of their growth and that of aggregated lymph-node cells.

It seems that the cancer bearing host recognizes own cancer cells as not-self and responds immunologically to cancer specific antigens through the lymphatic system. On the basis of this assumption, it is assumed that the lymph-node cells

in the host are specifically sensitized by the invading (or *in vivo*) antigen and form a sort of receptor having an immunological activity on the cell surface, which in turn comes in contact with the antigen on the surface of target cell, and this phenomenon is represented by the aggregation of lymph-node cells *in vitro*. It is deduced that since A-L cells (the regional sensitized lymph-node cells) obtained from the isologous C_3H mouse transplanted with A cells do markedly aggregate onto A cells, this target cell, A cell, has some antigen not possessed by C_3H mouse itself.

SUMMARY

As a link in the series of studies on tumor specific immunity an attempt was made to clarify specificity if any, in aggregation of sensitized lymph-node cells on target cell *in vitro*. For this purpose sensitized regional lymph-node cells from isologous C_3H mouse transplanted with A cells derived from C_3H mouse mammary cancer were incubated with M cells derived from mammary cancer of homologous Cb mouse and HeLa-S_3 cells as with A cells. The results are briefly summarized in the following.

These sensitized regional lymph-node cells (A-L) inhibited the proliferation of A cells and M cells in tissue culture. When the interaction between the sensitized lymph-node cells and the terget cells was pursued over a long period by cinematography, these lymph-node cells became attached to the target cell by 6- to 12-hour culture in aggregation of rosette form, and by 30 hours some of the target cells were seen to undergo lysis. However, when these sensitized lymph-node cells were cultured with heterologous HeLa-S_3 cells (derived from human uterine cancer), no such phenomena were observed. In the case with untreated normal lymph-node cells (control) there could be hardly observed any inhibitory effect on target cells.

When the number of the target cells on which the lymph-node cells became attached was counted along with lapse of time, it was more numerous in the case of A and M cells but only a few in the case of HeLa-S_3 cells.

It seems that most of the sensitized lymph-node cells that inhibit the growth of the target cells become attached and aggregated fairly specifically onto the target cells.

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